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(54) **HOST MICROORGANISMS**

(57) A microorganism wherein one or more genes selected from the group of genes participating in sporulation in the middle to late stages of sporulation have been deleted or inactivated; and a process for producing a target product (a protein) by use of the microorganism. No spore is formed when this microorganism is employed, thereby enabling production of a target product

(a protein) while decreasing energy loss, production of a by-product and specific production speed to decrease unnecessary consumption of a medium. Moreover, the production period can be prolonged, whereby the target product (the protein) can be produced efficiently.

**Description****Technical Field**

5 [0001] The present invention relates to a host microorganism which is useful for the production of useful proteins or polypeptides, and to a recombinant microorganism.

**Background Art**

10 [0002] Microorganisms have been employed in the industrial production of a broad range of useful substances. For example, microorganisms have been used to produce not only alcoholic beverages and foods such as *miso* (fermented soy paste) and *shoyu* (soy sauce), but also amino acids, organic acids, nucleic acid-related substances, antibiotics, sugars, lipids, proteins, and many other types of substances. Use of such substances can be found in a wide range of fields, encompassing foods, pharmaceuticals, daily necessities such as detergents and cosmetics, and a variety of raw materials for producing items through chemical processes.

15 [0003] One important issue in the industrial production of useful substances through use of microorganisms is improvement in productivity. Thus, as a measure therefor, cultivation of substance-producing microorganisms has been performed through traditional genetic techniques such as mutation. In particular, thanks to progress in microbial genetics and biotechnology, such cultivation of substance-producing microorganisms is now being carried out more efficiently than ever by use of a genetic technologies such as a genetic engineering technology, giving rise to development of host microorganisms useful for genetic recombination. For example, there has been developed a microorganism strain, which resulted from improvement of a microorganism strain *Bacillus subtilis* Marburg No. 168 that had been acknowledged to be safe and excellent.

20 [0004] Microorganisms harbor a diversity of genes, so that they can adapt themselves to environmental changes in the natural kingdom. Therefore, substance productivity of microorganisms cannot necessarily be said to be efficient in terms of industrial production of proteins or similar substances employing limited types of production medium.

25 [0005] Also, concerning certain types of microorganisms, there have been established strains in which genes participating in early stage sporulation are singly deleted or inactivated. However, these strains will not be said to be sufficiently improved in productivity.

30 [0006] Accordingly, an object of the present invention is to provide a host microorganism with which production of proteins or polypeptides can be increased through removal of genes which are useless or harmful in the production of proteins or polypeptides from the genome or inactivation of such genes. Another object of the present invention is to provide a recombinant microorganism produced by incorporating, into the above-mentioned host microorganism, a gene which codes for a protein or polypeptide and which is ligated to a transcription initiation regulation region, translation initiation regulation region, or secretion signal region at the downstream end of the region. Yet another object of the present invention is to provide a method for producing a protein or polypeptide by use of the recombinant microorganism.

**Disclosure of the Invention**

40 [0007] The present inventors have extensively searched, among a variety of genes encoded on a microorganism genome, for genes which are useless or function harmfully in the production of useful proteins or polypeptides, and have found that productivity of a protein or polypeptide of interest can be enhanced by deleting from the genome a specific gene participating in sporulation or by inactivating the gene, and then incorporating into the microorganism a gene which encodes a target protein or polypeptide and which has been ligated to a suitable transcriptional initiation region, a translational initiation region, or a secretion signal region, as compared with the productivity attained by the microorganism without such deletion or inactivation.

45 [0008] Accordingly, the present invention provides a microorganism in which one or more genes selected from the genes which participate in sporulation in the middle to late stages of sporulation have been deleted or inactivated; a recombinant microorganism obtained by incorporating, into the gene-deleted or gene-inactivated microorganism, a gene which encodes a protein or polypeptide and which has been ligated to a transcription initiation regulation region, a translation initiation regulation region, or a secretion signal region at the downstream end of the region; and a method for producing a protein or polypeptide through use of the recombinant microorganism.

**Best Mode for Carrying Out the Invention**

55 [0009] No limitations are imposed on the parental microorganism which is used to construct the microorganism of the present invention, so long as it has a gene which participates in sporulation. Preferably, the parental microorganism

is a spore-forming microorganism. The parental microorganism may be a wild type or a mutant. Specific examples include bacteria belonging to the genus *Bacillus* such as *Bacillus subtilis*, bacteria belonging to the genus *Clostridium*, and yeasts, with bacteria belonging to the genus *Bacillus* being preferred. Among them, *Bacillus subtilis* is particularly preferred, in view that its complete genome information has already been elucidated, that techniques of genetic engineering and genomic engineering have been established, and that bacteria belonging to the *Bacillus subtilis* have an ability to secrete proteins outside the cells.

[0010] Examples of the target protein or polypeptide which is produced by use of the microorganism of the present invention include enzymes which are useful for foodstuffs, drugs, cosmetics, detergents, fiber treatment, drugs for medical tests, etc.; and proteins and polypeptides such as physiological active factors.

[0011] Two hundred and fifty or more genes discretely present on the genome have been identified to take part in sporulation. Among them, a target gene to be deleted or inactivated in the present invention is preferably a gene that promotes sporulation, and examples of such a gene include those encoding a sporulation-stage-specific  $\sigma$ -factor, genes participating in expression of any of the  $\sigma$ -factor genes, and genes participating in activation of any of the  $\sigma$ -factors. In addition, genes which are transcribed by any of the  $\sigma$ -factors to thereby participate in promotion of sporulation are also included within the scope of the present invention. In the early stage of sporulation (stages 0 - I), extracellular enzymes such as proteases and amylases have been known to be produced in increased amounts as compared with the amounts produced in logarithmic growth phases. Therefore, a target gene to be deleted or inactivated is preferably one or more genes which are expressed specifically in the middle to late stages of sporulation to thereby participate in sporulation. Specifically, a target gene is preferably one or more genes involved in the sporulation stage II, III, IV, or V, more preferably stage II or III, particularly preferably stage II. The present inventors have found that these genes are not directly involved in production of proteins of interest and are also not required for growth of the microorganisms in ordinary medium for industrial production.

[0012] Such genes of *Bacillus subtilis* are listed in Tables 1 and 2.

[0013] In the present specification, names, sites, base numbers, and functions of the genes are described on the basis of the *Bacillus subtilis* genome database that has been reported in Nature, 390, 249-256 (1997) and also published by JAFAN: Japan Functional Analysis Network for *Bacillus subtilis* (BSORF DB) (<http://bacillus.genome.ad.jp/>).

Table 1

Gene	Site (kb)	Function
<i>sigE</i>	1,604	Stage II, mother cell-specific $\sigma$ E factor
<i>sigF</i>	2,443	Stage II, forespore-specific $\sigma$ F factor
<i>spolIISB</i>	1,328	Stage II and subsequent stages, participating in sporulation
<i>spolIE</i>	71	Stage II, activating forespore-specific $\sigma$ F factor
<i>sigG</i>	1,605	Stages III-V, forespore-specific $\sigma$ G factor
<i>spolIVCB - spolIIC</i>	2,652-2,701	Stages IV-V, mother cell-specific $\sigma$ K factor

Table 2

Gene	Site (kb)	Function
<i>spolIIGA</i>	1,604	Stage II, activating mother cell-specific $\sigma$ E factor
<i>spolIIGA</i>	2,444	Stage II, participating in activation of forespore-specific $\sigma$ F factor
<i>spolIVFB</i>	2,855	Stages IV-V, activating mother cell-specific $\sigma$ K factor
<i>SpolIIR</i>	3,794	Stage II, participating in activation of mother cell-specific $\sigma$ E factor
<i>SpolIIR</i>	4,213	Stages III-V, participating in activation of forespore-specific $\sigma$ G factor

[0014] The following genes are considered to be equivalent to the genes listed in Table 1: Genes having the same functions as those of the *Bacillus subtilis* genes listed in Table 1 or 2, and genes derived from other microorganisms, preferably derived from a bacterium which belongs to the genus *Bacillus*, and exhibiting 70% or more homology, preferably 80% or more, more preferably 90% or more homology, with one of the genes listed in Table 1. These genes are included in the genes which are to be deleted or inactivated according to the present invention. Homology between amino acid sequences is calculated through the Lipman-Pearson method (Science, 227, 1435 (1985)).

[0015] When one or more genes selected from among the genes described above are deleted or inactivated, chemical

energy consumption required for sporulation of the microorganism is reduced, production period of proteins or polypeptides is prolonged, or other benefits are obtained, resulting in improved productivity of the proteins or polypeptides.

5 [0016] No limitations are imposed on the number of the genes which are deleted or inactivated, so long as at least one gene is deleted or inactivated. The number may be three or more, or five or more. The number is preferably two or three, particularly preferably two.

[0017] In order to construct the microorganism of the present invention, one or more genes in addition to the above genes may be deleted or inactivated. Through such a combination, a greater effect in improvement of the productivity could be expected.

10 [0018] Deletion or inactivation of a gene can be performed through known methods. Examples include a method in which target genes are sequentially deleted or inactivated, and a method in which one or more arbitrary DNA fragments are deleted or mutated for inactivation and the resultant gene is analyzed and evaluated in terms of the protein productivity by means of a suitable technique.

15 [0019] A target gene is deleted or inactivated through, for example, a homologous recombination method. Specifically, a DNA fragment containing a target gene is obtained through cloning by use of a suitable plasmid vector. The obtained DNA fragment is mutated by, among other methods, deleting the entire region of the gene or a portion of the target gene region through a routine gene engineering technique while retaining the DNA fragments connected to the respective ends of the target gene; by causing a nonsense mutation in the structural gene through base substitution, frameshift mutation or the like; or by isolating the target gene fragment through cloning or PCR and inserting a DNA fragment into the isolated target gene fragment. Subsequently, the mutated DNA fragment is introduced into a parental microorganism, to thereby cause homologous recombination with the parental microorganism genome at both regions adjacent to the target gene at the respective ends thereof. Thus, the target gene on the genome can be substituted by a DNA fragment in which the target gene has been deleted or inactivated.

20 [0020] Several methods have been reported for deleting or inactivating a target gene through homologous recombination when a bacteria in *Bacillus subtilis* is employed as a parental microorganism for producing the microorganism of the present invention (e.g., Mol. Gen. Genet., 223, 268 (1990)). The host microorganism of the present invention can be obtained through repetition of such a method.

25 [0021] Deletion or inactivation of one or more arbitrary DNA fragments can also be performed by obtaining one or more arbitrary DNA fragments from a parental microorganism through cloning and performing homologous recombination by use of the fragments in a manner similar to that described above, or alternatively by radiation of a  $\gamma$ -ray to the parental microorganism.

30 [0022] The recombinant microorganism of the present invention can be obtained by incorporating a gene encoding a target protein or polypeptide (hereinafter referred to as "a target protein or polypeptide gene") to the thus-obtained microorganism (host microorganism) in which one or more genes selected from the genes participating in sporulation in the middle to late stages of sporulation have been deleted or inactivated.

35 [0023] No limitations are imposed on the target protein or polypeptide gene. Examples of such genes include industrially usable enzymes such as enzymes for producing detergents, food, fibers, feed, and chemicals, for medical use, and for diagnosis, and physiologically active peptides. The industrially usable enzymes may be classified, on the basis of their functions, into oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases/synthetases, and others. Preferred examples of the target protein or polypeptide gene include genes encoding a hydrolase such as a cellulase, an  $\alpha$ -amylase, or a protease. Specific examples include cellulases belonging to the family 5 in Classification of polysaccharide hydrolases (Biochem. J., 280, 309 (1991)). Among them, cellulases derived from a microorganism, particularly cellulases derived from a bacterium belonging to the genus *Bacillus* are illustrated. More specific examples include alkaline cellulases derived from a bacterium belonging to the genus *Bacillus* and having a sequence of SEQ ID NO: 1 or 2, and cellulases having a sequence having 70% or more, preferably 80% or more, more preferably 90% or more homology with the sequence of SEQ ID NO: 1 or 2. The homology between amino acid sequences is determined through the Lipman-Pearson method (Science, 227, 1435 (1985)). Examples of the  $\alpha$ -amylases include  $\alpha$ -amylases derived from a microorganism, and liquefaction-type amylases derived from a bacterium belonging to the genus *Bacillus* are particularly preferred. Examples of the proteases include serine proteases and metal proteases derived from a microorganism, particularly derived from a bacterium belonging to the genus *Bacillus*.

40 [0024] A target protein or polypeptide gene is desirably ligated, at the upstream end thereof, to a regulation region participating in transcription or translation of the gene or secretion of the gene product, i.e., a transcriptional initiation regulation region containing a promoter and a transcriptional initiation point, a translation initiation region containing a ribosome binding site and an initiation codon, or a secretion signal peptide region in a suitable form. For example, a target protein or polypeptide gene is desirably ligated to the above regulation regions contained in a cellulase gene derived from a bacterium belonging to the genus *Bacillus* which is described in, for example, Japanese Patent Application Laid-Open (*kokai*) No. 2000-210081 or Hei 4-190793, and the above regulation regions contained in a region adjacent to the cellulase gene at the upstream end of the gene and having a length of 1 kb or less, preferably 0.6 kb or less. Specifically, a target protein or polypeptide gene is desirably ligated to, among others, a sequence of SEQ ID

NO: 1 or 2, or a base sequence having a certain degree of homology with SEQ ID NO: 1 or 2 and having a regulation function similar to that described above.

[0025] The recombinant microorganism of the present invention can be obtained by combining a DNA fragment containing a target protein or polypeptide gene with a suitable plasmid vector and incorporating the recombinant plasmid into a host microorganism cell through a routine transformation method. Alternatively, the recombinant microorganism of the present invention can be obtained by using as the DNA fragment a DNA fragment ligated to a suitable homologous region of a host microorganism gene and incorporating the resultant DNA fragment directly into the host microorganism gene.

[0026] Production of a target protein or polypeptide through use of the recombinant microorganism of the present invention may be performed by inoculating the recombinant microorganism into a medium containing an assimilable source of carbon and nitrogen and the other essential components, culturing the recombinant microorganism through a conventional method, and, after completion of culture, collecting and purifying the target protein or polypeptide.

[0027] As described above, a host microorganism of interest in which a sporulation-related gene has been deleted or inactivated can be produced, and a recombinant microorganism of interest can be produced through use of the host microorganism. In addition, by use of the recombinant microorganism, a useful protein or polypeptide can be produced efficiently. An example case in which  $\alpha$ -amylase or cellulase is produced through use of *Bacillus subtilis* will next be specifically described.

[0028] For example, when the *sigF* gene of a bacterium *Bacillus subtilis* (768 bp) encoding an RNA polymerase subunit  $\sigma$ F-factor which expresses in a forespore in stage II or subsequent stages of sporulation is to be deleted, the following procedure may be employed.

[0029] In the first step, a genome gene is extracted from a host microorganism of a *Bacillus subtilis* strain. Using the genome gene as a template, a DNA fragment at the upstream of the initiation codon of the *sigF* gene and a DNA fragment at the downstream of the termination codon of the *sigF* gene are joined by a marker gene such as chloramphenicol resistant gene inserted therebetween through SOE (splicing by overlap extension) - PCR (Gene, 77, 61 (1989)) or other methods.

[0030] In the next step, the host bacterium *Bacillus subtilis* is transformed by use of the thus-obtained DNA fragment through a competent method, and the transformant is isolated on the basis of chloramphenicol resistance or other characteristics, to thereby cause homologous recombination in the upstream and downstream regions of the *sigF* gene to give a transformant in which the *sigF* gene on the genome is substituted by a marker gene such as a chloramphenicol resistant gene or the like.

[0031] Thereafter, into the thus-obtained transformant and the original cell line of *Bacillus subtilis* serving as a control, a plasmid containing a gene encoding  $\alpha$ -amylase or cellulase is introduced. The thus-obtained recombinant is incubated under suitable conditions, for example, under shaking in a vegetative medium. The supernatant of the culture solution is measured in terms of  $\alpha$ -amylase activity or cellulase activity, and its productivity is compared with that of the original cell line of *Bacillus subtilis*, to thereby confirm that an increased amount of the target product can be obtained by deleting the *sigF* gene. When the culture solution is subjected to an isolation and purification procedure,  $\alpha$ -amylase and cellulase can be obtained.

#### Examples

##### Example 1

[0032] A genomic DNA was extracted from *Bacillus subtilis* 168. Using the extracted gene as a template, a 1.5-kb DNA fragment (A), on the genome, adjacent to the *sigF* gene (Base No. 2442658  $\leftarrow$  2443425) at the upstream end thereof and a 1.5-kb DNA fragment (B) adjacent to the *sigF* gene at the downstream end thereof were multiplied. Separately, a 0.9-kb DNA fragment (C) containing a chloramphenicol resistant gene was multiplied using plasmid pC194 as a template. The fragments (A), (B), and (C) were serially ligated in this order, through SOE-PCR, to thereby prepare a 3.9-kb DNA fragment. The *Bacillus subtilis* 168 was transformed using the thus-obtained DNA fragment through a competent method. The transformed *Bacillus subtilis* 168 was cultured on an LB agar medium containing chloramphenicol, and the colonies were isolated as a transformant. The resulting transformant was confirmed through PCR and sequencing to have a genome in which the region containing the *sigF* gene (2442632-2443318) had been deleted and substituted by the chloramphenicol resistant gene. Separately, each of the following regions on the genome was deleted and substituted by the chloramphenicol resistant gene in a manner similar to that described above: a region (1604136-1604976) containing the *sigE* gene (1604166  $\rightarrow$  1604885), a region (1347781-1348081) containing a substantial part of the *spolIISB* gene (1347913  $\leftarrow$  1348083), a region (70537-73018) containing a substantial part of the *spolIIE* gene (70536  $\rightarrow$  73019), a region (1605083-1605877) containing a substantial part of the *sigG* gene (1605025  $\rightarrow$  1605807), a region (2652156-2652723) containing the *spolVCB* gene (2652262  $\rightarrow$  2652732), or a region (2652156-2701031) containing a region from the *spolVCB* gene to the *spolIIC* gene (2652262  $\rightarrow$  2701023), to thereby

5 prepare a microorganism in which a gene participating in sporulation is deleted.

10 [0033] A DNA fragment (3.1 kb) of the alkaline cellulase gene derived from *Bacillus* sp. KSM-S237 (Japanese Patent Application Laid-Open (*kokai*) No. 2000-210081) was introduced into a shuttle vector pHY300PLK at the cleavage point of restriction enzyme *BamHI*, to thereby prepare a recombinant plasmid pHY-S237. The plasmid was incorporated through the protoplast method into each of the gene-deleted microorganisms prepared in Example 1 and *Bacillus subtilis* 168 serving as a control. The thus-obtained microorganism was incubated overnight under shaking at 37°C in an LB medium (10 mL). The resultant culture solution (0.05 mL) was inoculated to 2 × L-maltose medium (50 mL; 2% tryptone, 1% yeast extraction, 1% NaCl, 7.5% maltose, 7.5-ppm manganese sulfate 4-5 hydrate, 15-ppm tetracycline), followed by incubation for three days under shaking at 30°C. After completion of incubation, the cells were removed from the culture solution through centrifugation, and the alkaline cellulase activity of the supernatant was measured to determine the amount of alkaline cellulase secreted outside the cells during incubation. As a result, as shown in Table 3, all of the microorganisms in which a gene participating in sporulation had been deleted were found to secrete an increased amount of alkaline cellulase as compared with the control microorganism *Bacillus subtilis* 168 (wild type).

15 Table 3

Deleted gene	Site of the gene (kb)	Amount of alkaline cellulase secreted (relative value)
<i>sigE</i>	1,604	217
<i>sigF</i>	2,443	212
<i>spolISB</i>	1,328	140
<i>spolIE</i>	71	216
<i>sigG</i>	1,605	163
<i>spolIVCB - spolIIC</i>	2,652-2,701	141
<i>spolIVCB</i>	2,652	141
None (wild type)	-	100

## 20 30 Industrial Applicability

25 [0034] When the microorganism of the present invention is employed, no spores are formed. Therefore, the invention enables production of a target protein or target polypeptide while decreasing energy loss, production of by-products and specific production speed to largely decrease unnecessary consumption of a medium. Moreover, the production period of the protein or polypeptide can be prolonged, whereby the target product can be produced efficiently.

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55

## SEQUENCE LISTING

5 <110> KAO CORPORATION

10 <120> Host microorganisms

15 <130> KS0660

15 <140>

15 <141>

20 <150> JP P2001-160520

20 <151> 2001-05-29

25 <160> 2

30 <170> PatentIn Ver. 2.1

35 <210> 1

35 <211> 3150

35 <212> DNA

40 <213> Bacillus sp. KSM-S237

45 <220>

45 <221> CDS

45 <222> (573)..(3044)

50 <220>

50 <221> sig\_peptide

50 <222> (573)..(659)

55 <220>

55 <221> mat\_peptide

55 <222> (660)..(3044)

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65 taaaaatcagg taaacaggcc cttttttttt tttttttttt tttttttttt acgtttttttt 120

70 gaaataaaat tagttttttttt aggtttttttt tttttttttt tttttttttt 180

75 gttttttttt tttttttttt acgtttttttt tttttttttt tttttttttt tttttttttt 240

80 aaaccctttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 300



	110	115	120	
5	gii cai gcg cca ggi gai cci aga gai cci gii tat gca ggi gci aaa			1073
	Val His Ala Pro Gly Asp Pro Arg Asp Pro Val Tyr Ala Gly Ala Lys			
	125	130	135	
10	gai ttc tii aga gaa aii gca gct ita tac cci aai aat cca cac aii			1121
	Asp Phe Phe Arg Glu Ile Ala Ala Leu Tyr Pro Asn Asn Pro His Ile			
	140	145	150	
15	att tat gag tta gcg aat gag ccg agt agt aat aai aat ggi gga gca			1169
	Ile Tyr Glu Leu Ala Asn Glu Pro Ser Ser Asn Asn Gly Gly Ala			
	155	160	165	170
20	ggg aii ccg aat aac gaa gaa ggi tgg aaa gcg gta aaa gaa tat gct			1217
	Gly Ile Pro Asn Asn Glu Glu Gly Trp Lys Ala Val Lys Glu Tyr Ala			
	175	180	185	
25	gat cca aii gta gaa ait gta cgt aaa agc ggi aat gca gat gac aac			1265
	Asp Pro Ile Val Glu Met Leu Arg Lys Ser Gly Asn Ala Asp Asp Asn			
	190	195	200	
30	att atc aii gii ggi agt cca aac tgg agt cag cgt ccg gac ita gca			1313
	Ile Ile Ile Val Gly Ser Pro Asn Trp Ser Gln Arg Pro Asp Leu Ala			
	205	210	215	
35	gct gat aat cca aii gat cac cat aca ait tat act gtt cac ttc			1361
	Ala Asp Asn Pro Ile Asp Asp His His Thr Met Tyr Thr Val His Phe			
	220	225	230	
40	tac act ggt tca cat gct tca act gaa agc tat ccg tct gaa act			1409
	Tyr Thr Gly Ser His Ala Ala Ser Thr Glu Ser Tyr Pro Ser Glu Thr			
	235	240	245	250
45	ccii aac tct gaa aga gga aac gta ait agt aac act cgt tat gcg tta			1457
	Pro Asn Ser Glu Arg Gly Asn Val Met Ser Asn Thr Arg Tyr Ala Leu			
	255	260	265	
50	gaa aac gga gta gcg gta ttt gca aca gag tgg gga acg agt caa gct			1505
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	270	275	280	
55	agt gga gac ggt ggi cci tac tii gai gaa gca gai gta tgg aii gaa			1553
	Ser Gly Asp Gly Gly Pro Tyr Phe Asp Glu Ala Asp Val Trp Ile Glu			
	285	290	295	

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	Phe Leu Asn Glu Asn Asn Ile Ser Trp Ala Asn Trp Ser Leu Thr Asn	
	300 305 310	
10	aaa aat gaa gta tct ggt gca ttt aca cca ttc gag tta ggt aag tct	1649
	Lys Asn Glu Val Ser Gly Ala Phe Thr Pro Phe Glu Leu Gly Lys Ser	
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	415 420 425	
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50	ggt gct gag aag ctt aca atg gal gtt att gtt gat gaa cca acg acg	2033
	Gly Ala Glu Lys Leu Thr Met Asp Val Ile Val Asp Glu Pro Thr Thr	
	445 450 455	
55	gta gct att gcg gcg att cca caa agt agt aaa agt gga tgg gca aat	2081
	Val Ala Ile Ala Ala Ile Pro Gln Ser Ser Lys Ser Gly Trp Ala Asn	
	460 465 470	
	cca gag cgt gct gtt cga gig aac gcg gaa gal ttt gtc cag caa acg	2129
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	475	480	485	490	
5	gac ggt aag tat aaa gct gga tta aca att aca gga gaa gat gct cct				2177
	Asp Gly Lys Tyr Lys Ala Gly Leu Thr Ile Thr Gly Glu Asp Ala Pro				
	495		500		505
10	aac cta aaa aat atc gct ttt cat gaa gaa gat aac aat atg aac aac				2225
	Asn Leu Lys Asn Ile Ala Phe His Glu Glu Asp Asn Asn Met Asn Asn				
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## Claims

1. A microorganism in which one or more genes selected from the genes which participate in sporulation in the middle to late stages of sporulation have been deleted or inactivated.
2. The microorganism as recited in claim 1, which is a bacterium belonging to the genus *Bacillus*.
3. The microorganism as recited in claim 2, wherein the bacterium belonging to the genus *Bacillus* is *Bacillus subtilis*.
4. The microorganism as recited in any of claims 1 through 3, wherein the genes are expressed in any of stages II, III, IV, and V of sporulation and thus participate in sporulation.
5. The microorganism as recited in any of claims 1 through 4, wherein the gene to be deleted or inactivated is selected from the group consisting of *sigE* gene, *sigF* gene, *spolIE* gene, *spolISB* gene, and *sigG* gene of *Bacillus subtilis*, genes which fall within a region from *spoIVCB* to *spolIC* of *Bacillus subtilis*, and genes equivalent to any one or more of these genes.
6. A recombinant microorganism obtained by incorporating, into the microorganism as recited in any of claims 1 through 5, a gene which encodes a protein or polypeptide and which has been ligated to a transcription initiation regulation region, a translation initiation regulation region, or a secretion signal region at the downstream end of the region.
7. The recombinant microorganism as recited in claim 6, wherein the transcriptional initiation regulation region, the translational initiation regulation region, or the secretion signal region is derived from a cellulase gene of a bacterium belonging to the genus *Bacillus* or from a 1 kb region extending upstream of the cellulase gene.
8. The recombinant microorganism as recited in claim 6 or 7, wherein the transcription initiation regulation region, the translation initiation regulation region, or the secretion signal region is derived from a cellulase gene having a sequence of SEQ ID NO: 1 or 2 or a sequence having a homology of 70% or more with the sequence of SEQ ID NO: 1 or 2.
9. A method of producing a protein or polypeptide through use of the microorganism as recited in any one of claims 6 through 8.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/05151

A. CLASSIFICATION OF SUBJECT MATTER  
Int.Cl<sup>7</sup> C12N1/21, C12N15/09, C12N9/42

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
Int.Cl<sup>7</sup> C12N1/21, C12N15/09, C12N9/42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS (DIALOG), WPI (DIALOG),  
SwissProt/PIR/GeneSeq/GenBank/EMBL/DDBJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 492274 A2 (Eniricerche Societa per Azioni), 13 October, 1992 (13.10.92), & US 6284490 B1 & JP 4-287686 A	<u>1-4</u> 5-9
X	KENNEY T.J., Moran C.P. Jr., Organization and regulation of an operon that encodes a sporulation- essential sigma factor in <i>Bacillus subtilis</i> ., <i>J.Bacteriol.</i> 1987, Jul.;169(7):3329-39	<u>1-5</u> 6-9
X	MIN K.T., Yudkin M.D., Activity of mutant sigma F proteins truncated near the C terminus., <i>J.Bacteriol.</i> 1992 Nov.;174(22):7144-8	<u>1-5</u> 6-9

 Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
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Date of the actual completion of the international search 26 July, 2002 (26.07.02)	Date of mailing of the international search report 13 August, 2002 (13.08.02)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP02/05151

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARAK I., Youngman P., SpoIIE mutants of <i>Bacillus subtilis</i> comprise two distinct phenotypic classes consistent with a dual functional role for the SpoIIE protein., <i>J.Bacteriol.</i> 1996 Aug.;178(16): 4984-9	<u>1-5</u> 6-9
X	TAKAMATSU H. et al., The <i>Bacillus subtilis</i> yabG gene is transcribed by SigK RNA polymerase during sporulation, and yabG mutant spores have altered coat protein composition., <i>J.Bacteriol.</i> 2000 Apr.; 182(7):1883-8	<u>1-5</u> 6-9
Y	JP 2000-210081 A (Kao Corp.), 02 August, 2000 (02.08.00), Par. Nos. [0010], [0014]; sequence No.1 (Family: none)	6-9
P,X	KIM J.H. et al., Construction of spore mutants of <i>Bacillus subtilis</i> for the development as a host for foreign protein production., <i>Biotechnology Letters</i> , June 2001, Vol.23, No.12, pages 999 to 1004	1-9

Form PCT/ISA/210 (continuation of second sheet) (July 1998)